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# EPS and water in biofilms

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# CHAPTER ONE

## GENERAL INTRODUCTION: THE TRANSITION FROM BACTERIAL ADHESION TO THE PRODUCTION OF EPS AND BIOFILM FORMATION

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## ABSTRACT

Planktonic life is dangerous for most bacterial strains and species and adhesion to surfaces is often considered a survival mechanism. Once adhering, a cascade of events is triggered that involves amongst others, the production of an EPS-matrix leading to the formation of what is generally called a "biofilm". In this chapter, we hypothesize about the way bacteria become aware of their adhering state. We consider that bacteria deform under the influence of the adhesion forces exerted upon them when adhering to a substratum surface. Surface enhanced fluorescence has convincingly demonstrated the existence of minor cell wall deformation upon adhesion that can act as a trigger for an adhering bacterium to start EPS-matrix production, as a hallmark of the transition between planktonic and sessile phenotypes. For staphylococci, EPS-matrix production appears related with the magnitude of the adhesion forces felt. The importance of the EPS-matrix is ubiquitously present throughout the entire process of biofilm development, from facilitating initial bacterial adhesion to maintaining biofilm structural integrity during growth and offering "back up" resources in case of nutrient depletion during biofilm aging. During the entire biofilm life cycle, EPS protects the biofilm against chemical attacks such as from antimicrobial treatment and through its viscoelastic properties, against mechanical stresses. The viscoelastic response of biofilms to external mechanical stresses can be modeled using three Maxwell elements representing the flow of water, more viscous EPS and bacteria repositioning in a deformed biofilm. Bacterial repositioning is more prominent in open structured biofilms than in more condensed ones with an impact on antimicrobial penetration and detachment. The main advantages of stress relaxation to determine biofilm structure and composition over microscopic techniques are that it yields quantitative data covering an area of several square millimeters. Biofilms are also slippery due to their EPS-matrix, but whether the slipperiness of biofilms serves any physiological role or not is unknown. Summarizing, this chapter reviews the events occurring during the transition from bacterial adhesion to EPS-matrix production and biofilm formation from a physico-chemical perspective, offering new concepts like bacterial adhesion force-sensing and cell wall deformation as a trigger for the development of sessile bacterial phenotypes.

## 1.1 INTRODUCTION

Biofilm formation is initiated by bacterial adhesion to a substratum surface. Bacteria adhere to surfaces because the nutrient concentration at a surface is usually higher than in suspension (1–3) and in addition, adhesion often serves as a survival strategy. Oral microorganisms for instance, need to adhere to oral hard or soft tissues in order to avoid being swallowed with saliva and being killed in the gastrointestinal tract. Marine bacteria adhere to ship hulls to have easy access to nutrients and find shelter against predation (4).

Upon adhesion, bacteria produce a matrix of extracellular polymeric substances (EPS), which consists of polysaccharides, proteins, nucleic acids and lipids in which bacteria embed themselves (Figure 1). EPS production is a survival strategy as well for the microorganisms, since it provides higher mechanical stability to the biofilm, as well as higher resistance to antimicrobials and protection against the host immune system (5). When biofilms form on biomaterials implanted or introduced in the human body, such as catheters or joint implants, antibiotic resistance increases up to 1000 times the resistance of planktonic bacteria (6–8). As a consequence, these biomaterial-associated infections can mostly only be treated by implant replacement, involving additional invasive surgery for the patient and high costs for the health care system (9). To prevent biofilm formation, reduction of initial bacterial adhesion has been the main aim of worldwide investigation (10–12). Promising options for decreasing the number of bacteria to surfaces are hydrophilic polymer brush coatings, acting as steric barriers that prevent direct bacterial contact with a surface. However, the approach of reducing bacterial adhesion has limitations, since even adhesion of a small number of bacteria can lead to biofilm formation (13). Moreover it can be doubted whether interference in bacterial adaptation to their adhering state would not be a better way to go, leaving bacteria in their susceptible planktonic state, also when adhering.

Hitherto, the mechanisms of bacterial adaptation to their adhering state are not completely unraveled. Bacteria have only limited possibilities to sense a surface. Since bacteria are known to respond differently to different biomaterials (14, 15), we have hypothesized that adhering bacteria sense a surface through cell wall deformation under the influence of the prevailing adhesion forces as a first step in their adaptation to an adhering state and the formation of a biofilm. In this chapter, we review the available literature on adhesion force sensing and cell wall deformation as well as the main techniques for studying cell wall deformation and adhesion force sensing. Also, we will highlight the role of EPS production as triggered through adhesion force sensing in biofilm formation. Further research aimed at modifying the bacterial response upon adhesion to a surface in order to block bacterial transition from a “planktonic” to a “biofilm” mode of growth may yield novel strategies to prevent biofilm formation.

## 1.2 THE TRANSITION FROM BACTERIAL ADHESION TO BIOFILM FORMATION

Adhesion of the first arriving bacteria on a substratum surface is of primary importance, as these initial colonizers provide the link between the substratum surface and the entire

biofilm. It has been hypothesized that upon first contact with a surface, these "linking" bacteria sense the surface (16) through the magnitude of the adhesion force exerted by the substratum. Therewith the adhering bacteria become aware of their adhering state as a result of membrane deformation occurring during adhesion (9). Membrane deformation is minor in most naturally occurring strains and species, since it is counteracted by the rigid peptidoglycan layer surrounding a bacterium, but still sufficient to allow communication between a bacterium and its environment.

The mechanism by which a bacterium becomes aware of its adhering state has been named "surface sensing". This term has been applied to motile bacteria, whose flagella can sense the surface so that a bacterium can choose between adhering to the surface or remaining in the planktonic state (16, 17). Other authors used the term "surface sensing" with regard to non-motile bacteria, to indicate the "sense of touch" that takes place before adhesion to the surface and provides a bacterium with information about the type of material it adheres to (18, 19).

The first steps in biofilm formation always involve adhesion and surface sensing. The transition from a planktonic to a biofilm mode of growth is characterized by gene upregulation or downregulation and by the production of EPS to make bacteria more resilient to different stresses and to promote irreversible adhesion. The biofilm phenotype that develops after adhesion to a surface is often referred to as the "sessile phenotype" (2). In the first ten minutes after contact with a surface, *Escherichia coli* was found to decrease its cell respiration level compared to planktonic bacteria (20) and this phenomenon has been interpreted as a strategy to ensure survival of adhering bacteria in the eventuality of oxidative stress conditions during the biofilm formation and growth (21). In motile bacteria, the sessile phenotype often involves the repression of flagellum synthesis, facilitating irreversible cell attachment on the surface (16, 22). In Caulobacter crescentus, adhesin production is stimulated by adhesion on a substratum surface, in order to create irreversible attachment (23). *Staphylococcus aureus* also shows an accumulation of adhesins in contact with a surface (18).

In the examples above, adhesion forces prevail in the range of the "interaction regime", as described in a regimented "three adhesion force" model (9). According to this model, the "interaction regime" describes changes in phenotypes that gradually occur with increasing adhesion forces. Weak adhesion forces are related to the planktonic regime in which surface sensing is very weak and bacteria behave like planktonic cells. Without the necessary changes in gene expression and subsequent production of EPS, these cells remain susceptible to antimicrobials. This regime occurs for hydrogels and polymer-brush coated surfaces (13, 24). In the lethal regime, strong adhesion forces lead to membrane stresses that deactivate the adhering bacteria and cause cell death. In *Bacillus subtilis* it was shown how mechanical stresses on the cell membrane provoked cell death without disrupting the membrane integrity of the cells (25). For this effect, the term "stress-deactivation" has been proposed and a number of studies have focused on this topic (26).

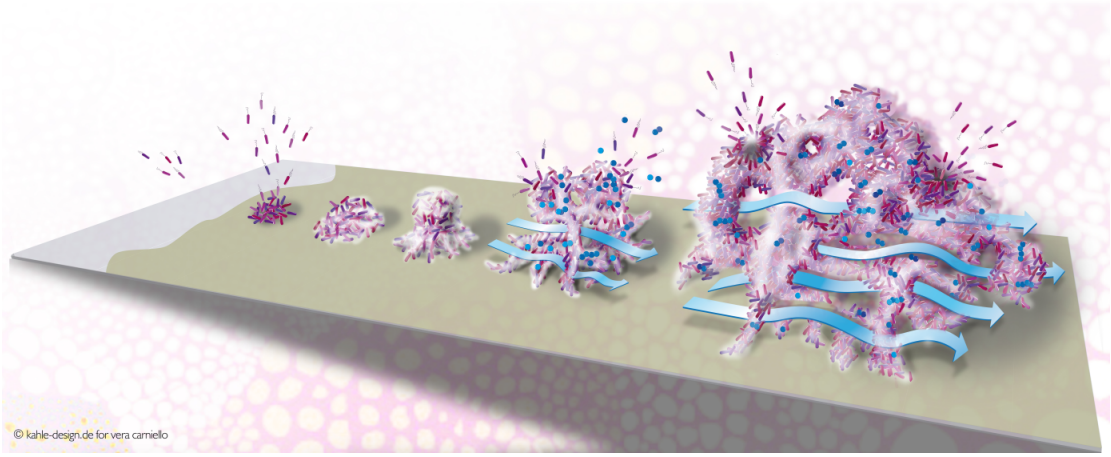


**Figure 1.** Steps in the transition from initial bacterial adhesion to biofilm formation. When a surface is in contact with body fluids, proteins and other compounds are adsorbed first onto the surface, forming the so-called "conditioning film" to which bacteria mostly adhere rather than to a bare substratum surface (depending environmental conditions). Upon adhesion to a surface, bacteria form a micro-colony by cell division and EPS production. Subsequently, colonies expand by adhesion of other bacteria, growth and the continued production of EPS (adapted from (27)).

## 1.3 BACTERIAL SURFACE SENSING AND CELL WALL DEFORMATION

Motile bacteria can sense surfaces through appendages like flagella or pili. The contact of these bacterial structures with a substratum surface causes a drag on the appendage and a subsequent local stress that provokes a signal inducing the transition to the biofilm phenotype (16). In *B. subtilis*, contact of a flagellum with a surface inhibits flagellar rotation and this surface signal activates the two-component regulatory system DegS–DegU that triggers the change in gene expression towards the sessile phenotype (28, 29).

In absence of surface appendages, non-motile bacteria sense the surface through cell wall deformation and the magnitude of adhesion forces. Following adhesion to a surface, cell wall deformation causes minor deformations of the lipid bilayer, provoking a change in intra-bilayer pressure profile in the lipid membrane (30). This variation in pressure profile can be sensed by the bacterial cell through gating of mechanosensitive channels or through stress sensitive proteins (31). Mechanosensitive channels represent an interesting response to surface adhesion and a mechanism for surface sensing. Cell wall deformation and variations in membrane curvature imply thinning of the membrane and wider spaces between lipids in the bilayer, which allow water molecules to insert within the lipids. This provokes energetically an unfavorable hydrophobic mismatch between lipids and water molecules and the energy necessary to compensate this is provided by the adhesion forces between bacterium and a substratum surface (31). Mechanosensitive channels gate in response to the variation in membrane pressure profile, and both membrane thinning and hydrophobic mismatch were found to facilitate channel gating (30, 32, 33). Mechanosensitive channel gating results in the opening of a pore in the bacterial membrane,

allowing a solute flux between the cytoplasm and the outer environment (34), which might be the surface signal for a bacterium to become aware of its adhering state.

Stress sensitive proteins are located on the bacterial membrane and trigger complex signaling pathways after adhesion on a substratum surface. In *E. coli* the outer membrane lipoprotein NlpE can sense surface adhesion and transmit the information to the stress sensitive protein CpxA (35). After detecting this signal, CpxA activates the Cpx pathway that regulates the expression of gene clusters related to the biofilm mode of growth (36, 37).

## 1.4 METHODS TO STUDY BACTERIAL CELL WALL DEFORMATION

Considering the role of cell wall deformation in making an adhering bacterium aware of its adhering state, we will now briefly review techniques to study bacterial cell wall deformation.

### 1.4.1 Macroscopic Bio-optical Fluorescence Imaging

Bio-optical fluorescent imaging is a macroscopic technique, suitable for *in vitro* as well as *in vivo* investigations. Its most relevant component is a highly sensitive CCD camera, which can measure the radiance from fluorescent probes. Images are obtained by trans-illumination or reflection at single or multiple excitation wavelengths (38).

Bio-optical fluorescence imaging can be used to analyze cell wall deformation, exploiting the effect of surface enhanced fluorescence (SEF). SEF involves an increase in fluorescence occurring when a fluorophore is close to a reflecting metal surface and it is due to two effects: surface plasmon resonance and a mirror effect (39). Surface plasmon resonance takes place when the absorption and emission spectra of the fluorophore overlap with the scattering spectrum of the metal surface and this resonance condition determines a greater radiance from the metal-fluorophore system. The mirror effect takes place when the fluorophore is illuminated by the Raman-scattered field reflected by the conducting metal. Under these conditions, the emitted intensity can be amplified up to four times (39, 40). The effect of SEF decreases exponentially with the distance between the fluorescent dye and the metal surface and the fluorescence enhancement is not detectable for distances larger than about 30 nm. For this reason SEF cannot only be exploited to measure the distance between a bacterium and a substratum surface (39), but also to measure bacterial cell wall deformation. After adhesion of a fluorescent bacterium on a reflecting metal surface, cell wall deformation will gradually bring more fluorophores within a bacterium closer to a substratum, i.e. in the range of SEF (Figure 2) (41) enhancing the fluorescence signal. The advantage of SEF over other techniques to measure cell wall deformation is that several millions of bacteria can be analyzed within one experiment, taking only several minutes.

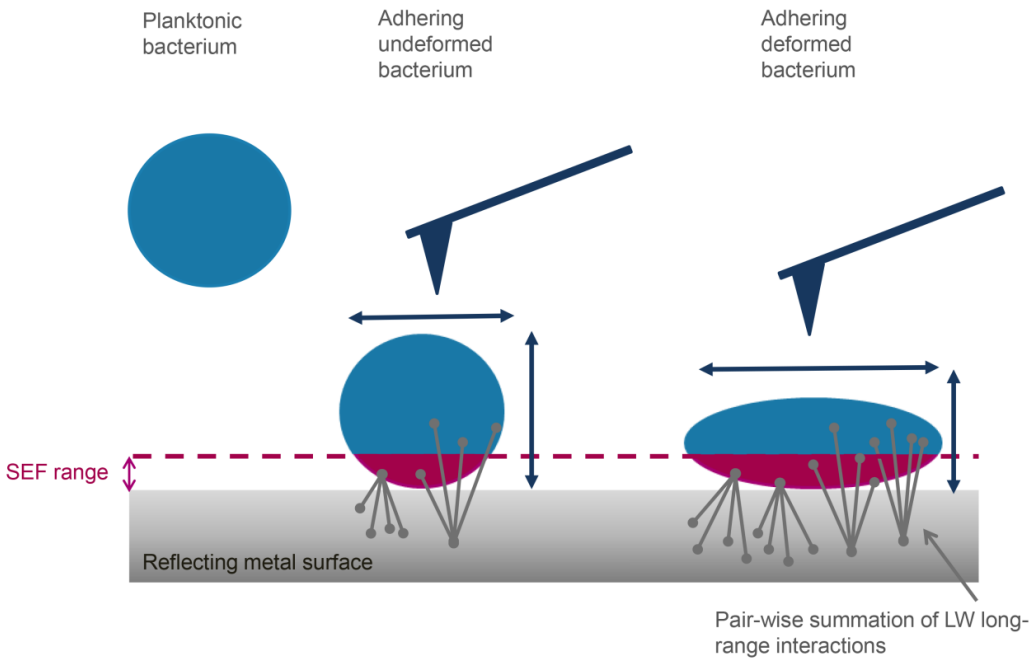


**Figure 2.** Lifshitz-Van der Waals interactions arise from the pair-wise summation of all possible molecular interactions between a bacterium and a substratum surface. Upon nanoscale cell wall deformation, more molecules inside a bacterium are brought in the close vicinity of a substratum, therewith increasing long-range Lifshitz-Van der Waals interactions (42). In case fluorescent bacteria are involved, more fluorophores are brought closer to a substratum surface upon cell wall deformation, i.e. within the range of SEF, and enhanced fluorescence can be measured with respect to undeformed bacteria. Deformation of individual bacteria can be measured using atomic force microscopy (AFM) imaging.

### 1.4.2 Atomic Force Microscopy

AFM allows to obtain topographical images of bacteria, cells or other particles and surfaces by scanning a sharp tip over the sample and measuring the interaction forces between the tip and the sample. This tip is mounted on a cantilever, which can be moved by a piezoelectric scanner in the x, y and z directions with high accuracy. When the cantilever bends in response to the interaction forces between tip and sample, the cantilever deflection is detected by the position of a laser beam reflected by the cantilever on a photodiode (43–46). AFM offers several modes for imaging living cells and microorganisms, as well as synthetic surfaces. In its contact mode, the tip is in direct contact with the sample and cantilever deflection is kept constant to maintain the same force between tip and sample at all times (47, 48). In tapping mode, the cantilever oscillates close to its resonance frequency, allowing the tip to come in contact with the sample at the end of its downwards movement (43, 49). Another mode of AFM application is PeakForce quantitative nanomechanical mapping (PeakForce-QNM) with which it is possible to simultaneously

record the topographical image as well as mapping other parameters of the sample, such as adhesion force and stiffness (50, 51).

AFM topographical imaging has been exploited to measure bacterial dimensions, i.e. height, width and length. Bolshakova and co-workers (52) employed AFM in both tapping and contact mode to measure the size of *E. coli* in air, water and bacterial culture medium. The authors concluded that the bacterial height measured in tapping mode in liquid was lower than in contact mode and that both height and width are smaller if the bacteria are dried to perform AFM measurements in air. Moreover, they observed that, when *E. coli* was treated with lysozyme, an enzyme that lyses the outer cell wall, the size of the Gram-negative bacteria decreased, their shape became more compressed and perforations of the inner cell membrane developed. Using the AFM in tapping mode, other authors found a significant height reduction in *E. coli* after treatment with the antibacterial drugs streptomycin and EDTA in combination with lysozyme (53). Similarly, measurement of the heights and widths of adhering bacteria can be done to quantify cell wall deformation after adhesion on a substratum surface (42, 54) using the AFM PeakForce-QNM imaging mode. By comparing the polar radius of individual, adhering bacteria with the radius of planktonic bacteria measured using dynamic light scattering the extent of cell wall deformation could be quantified and was found to be higher for Δpbp4 mutants lacking crosslinking of their peptidoglycan layer (42).

The major drawback of AFM imaging to investigate bacterial cell wall deformation is the need to measure individual, adhering bacteria, making it difficult to obtain statistically significant data. Furthermore, it can be argued that the loading force applied by the AFM cantilever can contribute to cell wall deformation. However, this drawback can be limited by using the PeakForce-QNM mode of AFM, which allows to image adhering bacteria by applying a low loading force (42).

### 1.4.3 Surface Thermodynamic Approach

The surface thermodynamic approach to bacterial adhesion is based on a surface free energy analysis of the bacterial and substratum surface along with the surface tension of the suspending liquid (55–57). Different methods to calculate substratum surface free energies exist using contact angle data with different liquids as input (58–60). Common to all methods is the Gibbs free energy of adhesion $\Delta G_{adh}$ as an outcome parameter, defined as:

$$\Delta G_{adh} = \gamma_{BS} - \gamma_{BL} - \gamma_{SL} \quad (1)$$

where $\gamma_{BS}$, $\gamma_{BL}$, $\gamma_{SL}$ are the interfacial free energies of the bacterium-surface, bacterium-liquid and surface-liquid interfaces, respectively (55, 56). Positive values of the Gibbs free energy of adhesion ($\Delta G_{adh} > 0$) correlate with unfavorable thermodynamic conditions for microorganisms to adhere on a surface, while energetically favorable conditions are characterized by $\Delta G_{adh} < 0$ (55).

The Gibbs free energy of adhesion can be divided in Lifshitz-Van der Waals and acid-base components (60):

$$\Delta G_{adh} = \Delta G_{adh}^{LW} + \Delta G_{adh}^{AB} \quad (2)$$

Lifshitz-Van der Waals interactions are due to attractive forces which are present as soon as a bacterium approaches a surface and can extend up to hundreds of nanometers (26, 61). The summation of all pair-wise Lifshitz-Van der Waals molecular interactions within a bacterium and a substratum surface gives rise to a long-range, attractive adhesion force between a bacterium and a substratum surface (Figure 2).

Even though bacteria have been demonstrated to adhere under conditions of unfavorable, positive values of the Gibbs free energy $\Delta G_{adh}$, the long-range Lifshitz-Van der Waals interactions $\Delta G_{adh}^{LW}$ are nearly always favorable for adhesion to occur ($\Delta G_{adh}^{LW} < 0$) and therewith generally considered responsible for adhesion (62). Moreover, it can be argued that since bacteria of different strains and species have a highly similar, chemical bulk composition, Lifshitz-Van der Waals components $\Delta G_{adh}^{LW}$ should be largely the same for bacteria adhering in an undeformed state. Since in a deformed state more molecules are brought in closer vicinity of a substratum surface, it has been speculated that differences in the Lifshitz-Van der Waals component $\Delta G_{adh}^{LW}$ are indicative of more or less extensive bacterial cell wall deformation (42), as explained in Figure 2.

## 1.5 BACTERIAL SURFACE SENSING AND THE ROLE OF EPS IN BIOFILMS

### 1.5.1 Bacterial Surface Sensing and EPS Production

EPS production after bacterial adhesion is one of the main hallmarks of the transition of adhering bacteria from a planktonic to a sessile phenotype and the formation of a biofilm. The analyses of surface sensing and subsequent EPS production have been mostly performed either from the perspectives of gene expression or through direct evaluation of the production of extracellular matrix components.

In adhering *S. aureus*, both icaA expression as well as production of poly-N-acetylglucosamine (PNAG), facilitating bacterial adhesion to surfaces and to other bacteria in multilayered biofilms (63), and eDNA decreased with increasing adhesion forces between adhering bacteria and substratum surfaces (64). This suggests that surface sensing through cell wall deformation is triggered by adhesion force sensing. The rigid peptidoglycan layer as part of the bacterial cell wall plays a pivotal role in counteracting adhesion force induced deformation, as in mutants lacking a rigid, cross-linked peptidoglycan layer no relation was found between adhesion forces and gene expression or matrix production (31, 64).

Another interesting technique to analyze gene expression and EPS production is β-galactosidase assay, a colorimetric assay that detects the enzyme β-galactosidase. This enzyme is encoded in *E. coli* by the gene lacZ, which can be fused to another gene of interest. Using a colorimetric assay that detects the enzyme β-galactosidase, the level of gene expression can be measured from the amount of β-galactosidase produced. In a *Pseudomonas aeruginosa* strain, it was found using this assay (65, 66), that fifteen minutes

after *Pseudomonas* adhesion to a surface, bacteria showed upregulated algC expression, governing the production of alginate as a matrix component.

### 1.5.2 Role of EPS in Biofilms

There are three distinctly different operational types of EPS, each with their own function: a. soluble EPS (sEPS), b. tightly bound EPS (TB-EPS) and c. loosely bound EPS (LB-EPS) (67).

sEPS molecules are much smaller than bacteria and once released, they will diffuse faster and adsorb to a substratum surface before initial bacterial adhesion (68). Adsorbed sEPS molecules can self-assemble and form hydrophilic patches that affect the hydrophobicity and charge properties of a substratum surface and promote bacterial adhesion. After the formation of a single, initial layer of adhering bacteria, the flexibility of sEPS patches helps to keep a favorable surface free energy state and thus allows new biofilm clusters to adhere (68). In mature biofilms, sEPS reduces its density by forming chain-like structures (69, 70). TB-EPS provides coherence to a biofilm bridging bacteria to each other to form clusters, while LB-EPS connects bacterial clusters into microcolonies (71). eDNA is a pivotal component of TB-EPS not only promoting bacterial adhesion to surfaces (71) but also co-adhesion between bacteria (72).

In a biofilm mode of growth, adhering bacteria tend to increase their EPS production and decrease their cell size to raise the probability of biofilm survival in case of nutrient depletion during aging of a biofilm (73). Also, during periods of low humidity, hydrophilic EPS enables water storage needed for survival. These survival strategies allow to increase the bacterial number by cell division while conserving the total amount of biomass (74). While during the early stages of biofilm formation (12 - 24 hours), large amounts of eDNA and polysaccharides are required to initiate biofilm formation (75, 76), during biofilm aging the major EPS components, such as polysaccharides, proteins and eDNA are accumulated and possibly become a “back up” nutrition source (73). In this phase of biofilm growth, eDNA helps to maintain the structural integrity of the biofilm through binding with other biofilm components and to protect its inhabitants against chemical and mechanical stress (77).

The chemical composition of EPS, and accordingly its physical properties, differs from place to place in a biofilm of the same strain. Growth medium, temperature, relative humidity, nutrition, pH and ionic strength have a major influence on EPS composition and physical properties and therewith function. EPS production is stimulated by low concentrations of nutrients because the matrix components can be used by the biofilm as a source of carbon, nitrogen and phosphorous in case of nutrient depletion (78). Low pH, high temperature and osmotic stress increase the EPS production, especially its protein content. This increases the resistance of *P. aeruginosa* in their biofilm mode of growth against heat stress and temperature-induced lysis (73).

### *1.5.2.1 EPS and resistance of biofilms against mechanical attack*

In most cases, biofilms do not grow in a static environment, but under conditions of fluid flow. Fluid flow and resulting shear forces influence bacterial adhesion and biofilm formation (79, 80), affecting deposition kinetics, architecture, dynamic behavior, physiological reaction and metabolic activities of a biofilm (81). EPS shows a different distribution and composition under different fluid shears in order to allow a bacterial community to persist under the environmental conditions of its formation. Biofilms growing under high flow rates appear to be thinner and more homogeneous (82) than biofilms formed under low flow rates. Also the prevalence of polysaccharides, proteins and TB-EPS components is increased in biofilm grown under fluid shear compared to its prevalence in biofilms grown under static conditions in order to provide more cohesion to the biofilm.

EPS provides viscoelastic properties to a biofilm (83, 84), that are pivotal for its survival under mechanical attack, such as experienced by oral biofilms during powered toothbrushing. Powered toothbrushing, particularly in absence of direct contact between the bristles and a biofilm, can be considered as a form of transmitting energy into the biofilm (85), upon which a biofilm expands. As for all viscoelastic materials, when expansion extends above the elastic limit of the biofilm, plastic deformation commences. Biofilm detachment subsequently results when expansion and plastic deformation continue, but when brushing is arrested while in the plastic range, a “fluffed-up”, very open biofilm will remain behind, i.e. the biofilm survives due to its viscoelastic properties (86).

### *1.5.2.2 EPS and resistance of biofilms against chemical attack*

Bacteria in their biofilm mode of growth have higher resistance to antimicrobials compared to their planktonic counterparts. EPS producing strains exhibit generally higher resistance against antibiotics than non-slime producing ones (87), as EPS reduces the penetration of antibiotics into the deeper layers of a biofilm and limits absorption of antibiotics to the outer layer of a biofilm. For *Acinetobacter baumannii* and *Staphylococcus aureus* this protection has been largely attributed to polysaccharides and lipids, because the biofilm resistance to antibiotics was not affected by selective removal of proteins or DNA from an EPS matrix (87). The chemical resistance of biofilms is usually illustrated as resulting from a combination of structure and composition, but evidence is based on cartoons and at best highly subjective microscopic images. Recently, the viscoelastic properties of biofilms have been advocated for use to reflect structure and composition of biofilm in a more quantitative way (88). The kinetics of stress relaxation of a biofilm after deformation showed that three processes mediate relaxation: a. fast flow of water in a deformed biofilm, b. intermediately flow of more viscous EPS and c. slow repositioning of bacteria provided sufficiently open space is available. Interestingly, biofilms that were demonstrated to be more open by stress relaxation analysis facilitated better penetration of antimicrobials (89).

#### *1.5.2.3 Lubricating properties of EPS in biofilms*

Biofilms are slippery due to their EPS matrix (90). Whether or not the slipperiness of biofilms serves any physiological role or not is unknown. Equally unknown is whether the lubricating properties of biofilm EPS, or for that matter bacteria themselves as ball-bearers, will ever find application. Yet, the lubricating properties of biofilms are another reflection of their structure and composition. Tribology measurements with AFM showed that biofilms on stainless steel of a slime producing *S. aureus* strain (*S. aureus* ATCC 12600) decreased the coefficient of friction (COF) of stainless steel to a much larger extent from 0.6 ± 0.3 to 0.1 ± 0.1 than the non-slime producing strain *S. aureus* 5298 (COF equals 0.3 ± 0.1).

## 1.6 METHODS TO STUDY BIOFILM COMPOSITION AND STRUCTURE

Considering the importance of biofilm structure and composition in resisting mechanical and chemical attacks, we will now briefly summarize different methods to investigate these properties.

### 1.6.1 Microscopic Structure of Biofilms

Using confocal laser scanning microscopy (CLSM), structural information can be acquired in fully hydrated biofilms, while also the distribution, shape, heterogeneity and structure of EPS can be observed (91). In order to provide an image, appropriate fluorescent dyes for EPS and bacteria are necessary. The staining procedure, however, causes two major limitations of CLSM imaging: a. A thick biofilm cannot be imaged due to the insufficient penetration of dyes and b. The biofilm cannot be reused for other studies after CLSM imaging because staining procedures cause irreversible harm to the bacteria.

As a powerful and convenient imaging tool, CLSM can be combined with for example, 3D-finite element analysis which can be constructed based on stacks of CLSM images (92). With the help of computer modeling, the signal pixels can be connected to each other to form a net structure, and subsequently a 3D biofilm model can be reconstructed from a series of 2D signals. The distribution and structure of EPS can be studied with fluorescence lectin-binding analysis (FLBA) (93, 94). FLBA is a non-destructive and in situ EPS analysis method since the chemical dyes used in CLSM imaging are replaced by fluorescence labeled lectin which has negligible influence on properties and functions of biofilms.

Biofilm structure can also be analyzed by optical coherence tomography (OCT) which is a completely non-destructive *in situ* technique (95).

### 1.6.2 Composition of Biofilms

Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy amongst other methods, allow determination of the chemical composition of biofilms grown on internal reflection elements e.g. a germanium crystal placed in a flow chamber. Both techniques provide spectra showing the quantitative prevalence of specific molecular groups in a hydrated biofilm in a non-destructive way (96–98). Extracellular substances such as polysaccharides, proteins, DNA and water, can be identified from the peaks in FTIR or Raman spectra, based on their specific peak wavenumber. Peak areas provide quantitative information on the amount of each extracellular substance. Spectra acquired at different stages of biofilm formation can be illustrated in a stack mode to show the variation of biofilm chemical composition during biofilm development.

Raman spectroscopy gives more or less similar information about biofilms as FTIR. Compared to FTIR spectroscopy however, Raman spectroscopy has several advantages: a. a weaker signal of water and $CO_2$, b. wider spectral range especially for wavenumbers below 400 $cm^{-1}$ and c. stronger signal for symmetric bonds (e.g. -S-S-, -C-S-, -C≡C-) (99–101).

### 1.6.3 Viscoelastic Properties of Biofilms

Viscoelastic properties of biofilms can be determined under shear, tensile or compressive conditions. Low load compression testing (LLCT) is one of the methods to determine stress relaxation after compression (88). Stress relaxation of biofilms can be modeled using three Maxwell elements representing the flow of water, more viscous EPS and bacteria repositioning in a deformed biofilm. Bacterial repositioning is more prominent in “fluffed up” biofilms than in more condensed ones. The main advantages of stress relaxation to determine biofilm structure and composition are that a. it yields quantitative data and b. the plunger applied e.g. in LLCT, covers an area of several square millimeters, while microscopic imaging can cover only a small fraction of this, often user-selected. A cone/plate rheometer is another method to determine the viscoelastic properties of a biofilm. It basically consists of a rotatable cone on top and a fixed plate at the bottom, with viscoelastic samples, e.g. biofilms in the middle. The cone can be set into a rotating or oscillating mode while sensors at the bottom plate measure the viscous or elastic property of the samples, respectively. However, when using rheological methods, biofilms are regarded as homogeneous, soft materials, and output parameters represent average values of the biomass as a whole (102). Information on possible micro-scaled structural heterogeneities in a biofilm are neglected. To address this drawback, Hohne et al. (103) developed a flexible microfluidic device in order to study the micro-scaled elastic moduli and the stress relaxation of specified parts in a biofilm. Biofilm grown on a silicon wafer were covered with a thin poly(dimethyl siloxane) film and deformation of the poly(dimethyl siloxane) film was induced with a controlled air pressure. The strain and stress relaxation time of biofilms were evaluated based on the deformation of the poly(dimethyl siloxane) film.

### 1.6.4 Lubricating Properties of Biofilms with and without EPS

Biofilm tribology is still in its infancy (104, 105). Coefficients of friction of biofilms can be measured by using a sliding wear tester. In a sliding wear test, a fixed stainless steel roller with a typical diameter of 4 mm is slided over a biofilm and the friction force generated is detected by a sensor connected to the roller-holder. On a more microscopic level, colloidal-probe AFM can be applied and such COF measurements are based on the torsion of the cantilever upon the friction between a biofilm and a silica ball, with a typical diameter of 30 µm. Often however, macroscopic and microscopic friction measurements of the COF of biofilms yield different results in terms of standard deviations and values found, as shown in Figure 3. The larger standard deviations observed with AFM are due to the microscopic nature of this technique, which is highly sensitive to small heterogeneities of both biofilms and the underlying surface, in contrast to the sliding wear tester which measures over a much larger surface area. The EPS producing strain (*S. aureus* ATCC 12600) shows in both cases the lowest coefficient of friction compared to the non-EPS producing strain (*S. aureus* 5298).

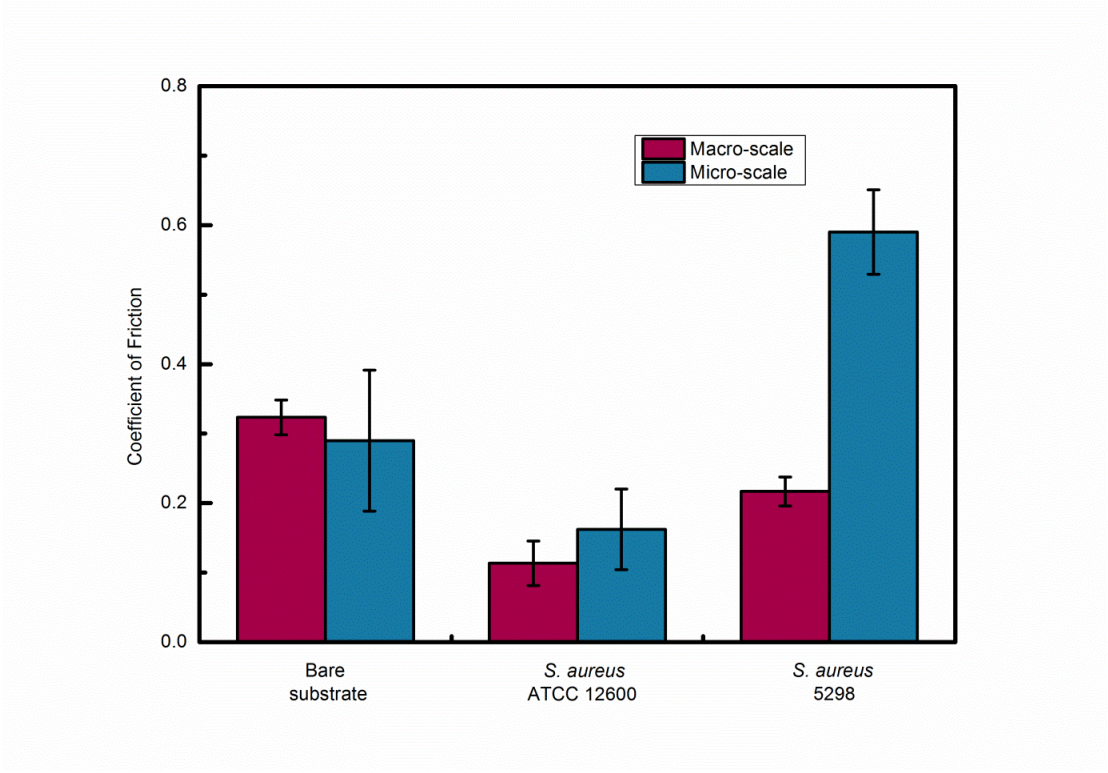


**Figure 3.** Coefficients of friction of staphylococcal biofilms on polyethylene of a EPS producing (*S. aureus* ATCC 12600) and non-EPS producing staphylococcal strain (*S. aureus* 5298), measured by a macroscopic sliding wear tester and microscopic AFM.

## 1.7 CONCLUSION

In this chapter, we reviewed the main steps in biofilm formation, starting from bacterial adhesion to surfaces. Upon initial adhesion of bacteria to a surface, the forces between a bacterium and the surface play an essential role in inducing nanoscale deformation of the bacterial cell wall including the lipid membrane. Membrane deformation eventually allows communication between an adhering bacterium and its external environment, making it aware of its adhering state. One of the main bacterial responses to surface sensing, and hallmark of the transition between planktonic and sessile phenotypes, is the production of EPS that form the biofilm matrix. The importance of EPS is ubiquitously present throughout the entire process of biofilm development, from facilitating initial bacterial adhesion to maintaining biofilm structural integrity during growth and offering “back up” resources in case of nutrient depletion during biofilm aging. During the entire biofilm life cycle, EPS protects the biofilm against chemical attacks such as from antibiotic treatment and through its viscoelastic properties, against mechanical external attacks.

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## AIM OF THE THESIS

A biofilm is not just an accumulation of bacterial cells but instead forms a community inside a matrix with EPS and water surrounding the bacteria for regulation of function and environmental stress protection. EPS and water are the main components of the biofilm matrix.

Therefore, the aim of this thesis is to study the fundamental mechanisms of biofilm resistance to physical stress and the role of EPS and water. To this end, both microscopic and spectroscopic methods were applied on biofilms of different bacterial strains in order to study the distribution, structure and function of EPS and water in a biofilm under various environmental stress conditions.